NUCLEOTIDE SEQUENCES WHICH CODE FOR THE deaD GENE

[0001] BACKGROUND OF THE INVENTION

The invention provides nucleotide sequences from coryneform bacteria which code for the deaD gene and a process for the fermentative preparation of amino acids using bacteria in which the deaD gene is attenuated. All references cited herein are expressly incorporated by reference. Incorporation by reference is also designated by the term "*I.B.R.*" following any citation.

[0002] L-Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

[0003] It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

[0004] Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner.

[0005] Methods of the recombinant DNA technique have also been employed for some years for improving the strain of

Corynebacterium strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

[0006] The invention provides new measures for improved fermentative preparation of amino acids.

[0007] BRIEF SUMMARY OF THE INVENTION

Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine is particularly preferred.

[0008] When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

[0009] The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the deaD gene, chosen from the group consisting of

a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of DNA/RNA helicase.

[0010] The invention also provides the above-mentioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

(i) the nucleotide sequence, shown in SEQ ID No.1, or

(ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

[0011] The invention also provides:

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No.1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing parts of the polynucleotide according to the invention, but at least 15 successive nucleotides of the sequence claimed,

and coryneform bacteria in which the deaD gene is attenuated, in particular by an insertion or deletion.

[0012] The invention also provides polynucleotides, which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

[0013] BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Map of the plasmid pXK99E,

Figure 2: Map of the plasmid pXK99EdeaD.

[0014] The abbreviations and designations used have the following meaning.

Kan:	Kanamycin resistance gene aph(3')-IIa from Escherichia coli
BstEII	Cleavage site of the restriction enzyme BstEII
HindIII	Cleavage site of the restriction enzyme HindIII
NcoI	Cleavage site of the restriction enzyme NcoI
XbaI	Cleavage site of the restriction enzyme XbaI
Ptrc	trc promoter
T1	Termination region T1
T2	Termination region T2

LacIq	LacIq repressor of the lac operon of Escherichia coli
OriV	Replication origin ColE1 from E. coli
DeaD	Cloned region of the deaD gene

[0015] DETAILED DESCRIPTION OF THE INVENTION

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for DNA/RNA helicase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence of the deaD gene. They can also be attached as a probe to so-called "arrays", "micro arrays" or "DNA chips" in order to detect and to determine the corresponding polynucleotides or sequences derived therefrom, such as e.g. RNA or cDNA.

[0016] Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for DNA/RNA helicase can be prepared by the polymerase chain reaction (PCR).

[0017] Such oligonucleotides which serve as probes or primers comprise at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, very particularly preferably at least 15, 16, 17, 18 or 19 successive nucleotides. Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100,

150, 200, 250 or 300 nucleotides are optionally also suitable.

[0018] "Isolated" means separated out of its natural environment.

[0019] "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

[0020] The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

[0021] "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

[0022] The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of DNA/RNA helicase and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

[0023] The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular

already produce amino acids and in which the nucleotide sequences which code for the deaD gene are attenuated, in particular eliminated or expressed at a low level.

[0024] The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

[0025] By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

[0026] The microorganisms provided by the present invention can prepare amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus Corynebacterium. Of the genus Corynebacterium, there may be mentioned in particular the species Corynebacterium glutamicum, which is known among experts for its ability to produce L-amino acids.

[0027] Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum (C. glutamicum), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539

Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom.

[0028] The new deaD gene from C. glutamicum which codes for the enzyme DNA/RNA helicase has been isolated.

[0029] To isolate the deaD gene or also other genes of C. glutamicum, a gene library of this microorganism is first set up in Escherichia coli (E. coli). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990) *I.B.R.*, or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) *I.B.R.* may be mentioned as an example. A well-known gene library is that of the E. coli K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495 -508 (1987)) *I.B.R.* Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) *I.B.R.* describe a gene library of C. glutamicum ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164 *I.B.R.*) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575 *I.B.R.*).

[0030] Börmann et al. (Molecular Microbiology 6(3), 317-326)) (1992)) *I.B.R.* in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, 1980, Gene 11, 291-298 *I.B.R.*).

[0031] To prepare a gene library of C. glutamicum in E. coli it is also possible to use plasmids such as pBR322 (Bolivar, 1979, Life Sciences, 25, 807-818 *I.B.R.*) or pUC9 (Vieira et al., 1982, Gene, 19:259-268 *I.B.R.*). Suitable

hosts are, in particular, those E. coli strains which are restriction- and recombination-defective, such as, for example, the strain DH5αmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) *I.B.R.* The long DNA fragments cloned with the aid of cosmids or other λ vectors can then in turn be subcloned and subsequently sequenced in the usual vectors which are suitable for DNA sequencing, such as is described e. g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977) *I.B.R.*

[0032] The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)) *I.B.R.*, that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) *I.B.R.* or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)) *I.B.R.*

[0033] The new DNA sequence of C. glutamicum which codes for the deaD gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the deaD gene product is shown in SEQ ID No. 2.

[0034] Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus

of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)) *I.B.R.*, in O'Regan et al. (Gene 77:237-251 (1989)) *I.B.R.*, in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)) *I.B.R.*, in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) *I.B.R.* and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

[0035] In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

[0036] Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) *I.B.R.* and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)) *I.B.R.* The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996 *I.B.R.*).

[0037] A 5x SSC buffer at a temperature of approx. 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995 *I.B.R.*) a temperature of approx. 50°C - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature stepwise from 50°C to 68°C in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

[0038] Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) *I.B.R.* and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994) *I.B.R.*

[0039] It has been found that coryneform bacteria produce amino acids in an improved manner after attenuation of the deaD gene.

[0040] To achieve an attenuation, either the expression of the deaD gene or the catalytic properties of the enzyme protein can be reduced or eliminated. The two measures can optionally be combined.

[0041] The reduction in gene expression can take place by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information on this e.g. in WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)) *I.B.R.*, in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998) *I.B.R.*, in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)) *I.B.R.*, in Pátek et al. (Microbiology 142: 1297 (1996)) *I.B.R.*, Vasicova et al. (Journal of Bacteriology 181: 6188 (1999)) *I.B.R.* and in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) *I.B.R.* or that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) *I.B.R.*

[0042] Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)) *I.B.R.*, Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) *I.B.R.* and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research Center, Jül-2906, ISSN09442952, Jülich, Germany, 1994) *I.B.R.* Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986) *I.B.R.*

[0043] Possible mutations are transitions, transversions, nsertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense

mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence of which incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) *I.B.R.*, that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990 *I.B.R.*) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986) *I.B.R.*

[0044] A common method of mutating genes of C. glutamicum is the method of "gene disruption" and "gene replacement" described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)) *I.B.R.*

[0045] In the method of gene disruption a central part of the coding region of the gene of interest is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)) *I.B.R.*, pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994) *I.B.R.*), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992) *I.B.R.*), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84 *I.B.R.*; US Patent 5,487,993 *I.B.R.*), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993) *I.B.R.*) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516 *I.B.R.*). The plasmid vector which contains the central part of the coding region of the gene is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is

described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)) *I.B.R.* Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)) *I.B.R.*, Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) *I.B.R.* and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)) *I.B.R.* After homologous recombination by means of a "cross-over" event, the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are obtained, one lacking the 3' end and one lacking the 5' end. This method has been used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) *I.B.R.* to eliminate the recA gene of C. glutamicum.

[0046] In the method of "gene replacement", a mutation, such as e.g. a deletion, insertion or base exchange, is established in vitro in the gene of interest. The allele prepared is in turn cloned in a vector which is not replicative for C. glutamicum and this is then transferred into the desired host of C. glutamicum by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation or of the allele is achieved. This method was used, for example, by Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)) *I.B.R.* to eliminate the pyc gene of C. glutamicum by a deletion.

[0047] A deletion, insertion or a base exchange can be incorporated into the deaD gene in this manner.

[0048] In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid

cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the attenuation of the deaD gene.

[0049] The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for a corresponding enzyme (protein) having a high activity, and optionally combining these measures.

[0050] By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

[0051] Thus, for the preparation of L-amino acids, in addition to the attenuation of the deaD gene at the same time one or more of the genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335 *I.B.R.*),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 *I.B.R.*),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 *I.B.R.*),

- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086 *I.B.R.*),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661 *I.B.R.*),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609 *I.B.R.*),
- the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998) *I.B.R.*),
- the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527 *I.B.R.*; EP-A-0699759 *I.B.R.*),
- the lysE gene which codes for lysine export (DE-A-195 48 222 *I.B.R.*),
- the hom gene which codes for homoserine dehydrogenase (EP-A 0131171 *I.B.R.*),
- the ilvA gene which codes for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072) *I.B.R.*) or the ilvA(Fbr) allele which codes for a "feed back resistant" threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842 *I.B.R.*),
- the ilvBN gene which codes for acetohydroxy-acid synthase (EP-B 0356739 *I.B.R.*),
- the ilvD gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979 *I.B.R.*),
- the zwa1 gene which codes for the Zwa1 protein (DE: 19959328.0 *I.B.R.*, DSM 13115)

can be enhanced, in particular over-expressed.

[0052] It may furthermore be advantageous for the production of amino acids, in addition to the attenuation of the deaD gene, at the same time for one or more of the genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1 *I.B.R.*, DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478 *I.B.R.*, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE:1995 1975.7 *I.B.R.*, DSM 13114),
- the zwa2 gene which codes for the Zwa2 protein (DE: 19959327.2 *I.B.R.*, DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced.

[0053] In addition to the attenuation of the deaD gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982 *I.B.R.*).

[0054] The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991) *I.B.R.*) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/ Wiesbaden, 1994)) *I.B.R.*

[0055] The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981) *I.B.R.*

[0056] Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

[0057] Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

[0058] Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

[0059] Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as, for example, antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for example, air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

[0060] Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) *I.B.R.* by anion exchange chromatography with subsequent ninhydrin derivation, or it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174) *I.B.R.*

[0061] The process according to the invention is used for fermentative preparation of amino acids.

[0062] The following microorganism was deposited as a pure culture on 22nd August 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Escherichia coli Top10/pXK99EdeaD as DSM 14464.

[0063] The present invention is explained in more detail in the following with the aid of embodiment examples.

[0064] The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA) *I.B.R.* Methods for transformation of Escherichia coli are also described in this handbook.

[0065] The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

[0066] **Example 1**

Preparation of a genomic cosmid gene library from C. glutamicum ATCC 13032

[0067] Chromosomal DNA from C. glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) *I.B.R.* and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250 *I.B.R.*). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences, USA 84:2160-2164 *I.B.R.*), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

[0068] The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase

(Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217 *I.B.R.*).

[0069] For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575 *I.B.R.*) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor) *I.B.R.*, the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

[0070] **Example 2**

Isolation and sequencing of the deaD gene

[0071] The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

[0072] The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-

01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor) *I.B.R.*, the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol. Letters, 123:343-7 *I.B.R.*) into the E. coli strain DH5αmcr (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649 *I.B.R.*). Letters, 123:343-7 *I.B.R.*) and plated out on LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 50 µg/ml zeocin.

[0073] The plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467 *I.B.R.*) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067) *I.B.R.* The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

[0074] The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231 *I.B.R.*) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analyses were prepared with the XNIP program (Staden, 1986,

Nucleic Acids Research, 14:217-231 *I.B.R.*). Further analyses can be carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402 *I.B.R.*) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA) *I.B.R.*

[0075] The relative degree of substitution or mutation in the polynucleotide or amino acid sequence to produce a desired percentage of sequence identity can be established or determined by well-known methods of sequence analysis. These methods are disclosed and demonstrated in Bishop, et al. "DNA & Protein Sequence Analysis (A Practical Approach"), Oxford Univ. Press, Inc. (1997) *I.B.R.* and by Steinberg, Michael "Protein Structure Prediction" (A Practical Approach), Oxford Univ. Press, Inc. (1997) *I.B.R.*

[0076] The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1875 bp, which was called the deaD gene. The deaD gene codes for a polypeptide of 624 amino acids.

[0077] Example 3

Preparation of the expression vector pXK99EdeaD for IPTG-induced expression of the deaD gene in C. glutamicum

[0078] 3.1 Cloning of the deaD gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) *I.B.R.* On the basis of the sequence of the deaD gene known for C. glutamicum from example 2, the following oligonucleotides were chosen for the polymerase chain reaction (see SEQ ID No. 3 and SEQ ID No. 4):

deaD for2:
5`-GA TCT AGA-AAT CCG GCT TCG ATG CAC GC-3` SEQ ID NO: 3

deaD int2:
5`- CT AAG CTT-CGA CGG TTG GCA GTT CCA TT-3` SEQ ID NO: 4

[0079] The primers were chosen here so that the amplified fragment contains the incomplete gene, starting with the native ribosome binding site without the promoter region, and the front region of the deaD gene. Furthermore, the primer deaD for2 contains the sequence for the cleavage site of the restriction endonuclease XbaI, and the primer deaD int2 the cleavage site of the restriction endonuclease HindIII, which are marked by underlining in the nucleotide sequence shown above.

[0080] The primers shown were synthesized by MWG-Biotech AG (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) *I.B.R.* with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment 1132 bp in size, which carries the incomplete deaD gene, including the native ribosome binding site.

[0081] The deaD fragment 1132 bp in size was cleaved with the restriction endonucleases XbaI and HindIII and then isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

[0082] 3.2 Construction of the expression vector pXK99E

The IPTG-inducible expression vector pXK99E was constructed according to the prior art. The vector is based on the Escherichia coli expression vector pTRC99A (Amann et al., Gene 69: 301-315 (1988) *I.B.R.*) and contains the trc promoter, which can be induced by addition of the lactose derivative IPTG (isopropyl β-D-thiogalactopyranoside), the termination regions T1 and T2, the replication origin ColE1 from E. Coli, the $lacI^q$ gene (repressor of the lac operon

from E.coli), a multiple cloning site (mcs) (Norrander, J.M. et al. Gene 26, 101-106 (1983) *I.B.R.*) and the kanamycin resistance gene aph(3´)-IIa from E. coli (Beck et al. (1982), Gene 19: 327-336 *I.B.R.*).

[0083] It has been found that the vector pXK99E is quite specifically suitable for regulating the expression of a gene, in particular effecting attenuated expression in coryneform bacteria. The vector pXK99E is an E. coli expression vector and can be employed in E. coli for enhanced expression of a gene.

[0084] Since the vector cannot replicate independently in coryneform bacteria, this is retained in the cell only if it is integrated into the chromosome. The peculiarity of this vector here is the use for regulated expression of a gene after cloning of a gene section from the front region of the corresponding gene in the vector containing the start codon and the native ribosome binding site, and subsequent integration of the vector into coryneform bacteria, in particular C. glutamicum. Gene expression is regulated by addition of metered amounts of IPTG to the nutrient medium. Amounts of 0.5 µM/l up to 10 µM/l IPTG have the effect of very weak expression of the corresponding gene, and amounts of 10 µM/l up to 100 µM/l have the effect of a slightly attenuated to normal expression of the corresponding gene.

[0085] The E. coli expression vector pXK99E constructed was transferred by means of electroporation (Tauch et al. 1994, FEMS Microbiol Letters, 123: 343-347 *I.B.R.*) into E. coli DH5αmcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649 *I.B.R.*). Selection of the transformants was carried out on LB Agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989 *I.B.R.*), which had been supplemented with 50 mg/l kanamycin.

[0086] Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927 *I.B.R.*), cleaved with the restriction endonuclease NcoI, and the plasmid was checked by subsequent agarose gel electrophoresis.

[0087] The plasmid construct obtained in this way was called pXK99E (figure 1). The strain obtained by electroporation of the plasmid pXK99E in the E. coli strain DH5αmcr was called E. coli DH5alphamcr/pXK99E (= DH5αmcr/pXK99E) and deposited on 31st July 2001 as DSM 14440 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

[0088] 3.3 Cloning of the deaD fragment in the E. coli expression vector pXK99E

The E. coli expression vector pXK99E described in example 3.2 was used as the vector. DNA of this plasmid was cleaved completely with the restriction enzymes XbaI and HindIII and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

[0089] The deaD fragment approx. 1120 bp in size described in example 3.1, obtained by means of PCR and cleaved with the restriction endonucleases XbaI and HindIII was mixed with the prepared vector pXK99E and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation batch was transformed in the E. coli strain DH5αmcr (Hanahan, In: DNA cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA *I.B.R.*). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190 *I.B.R.*) with 50 mg/l kanamycin. After

incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzymes XbaI and HindIII to check the plasmid by subsequent agarose gel electrophoresis. The resulting plasmid was called pXK99EdeaD. It is shown in figure 2.

[0090] Example 4

Integration of the vector pXK99EdeaD into the genome of the C. glutamicum strain DSM5715

[0091] The vector pXK99EdeaD mentioned in example 3 was electroporated by the electroporation method of Tauch et al.,(1989 FEMS Microbiology Letters 123: 343-347) *I.B.R.* in the strain C. glutamicum DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome. Selection of clones with integrated pXK99EdeaD was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989 *I.B.R.*), which had been supplemented with 15 mg/l kanamycin and IPTG (1mM/l).

[0092] For detection of the integration, the deaD fragment was labeled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993 *I.B.R.*). Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) *I.B.R.* and in each case cleaved with the restriction enzymes BstEII and XbaI. The fragments formed were separated by means of agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. The plasmid pXK99EdeaD

mentioned in example 3 had been inserted into the chromosome of DSM5715 within the chromosomal deaD gene. The strain was called DSM5715::pXK99EdeaD.

[0093] **Example 5**

Preparation of lysine

[0094] The C. glutamicum strain DSM5715::pXK99EdeaD obtained in example 4 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined. By addition of IPTG, attenuated expression of the deaD gene occurs, regulated by the trc promoter.

[0095] For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l) and IPTG (10 µM/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium Cg III was used as the medium for the preculture.

[0096]

Medium Cg III	
NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Kanamycin (25 mg/l) and IPTG (10 µM/l) were added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. The OD (660 nm) of the preculture was 0.8. 450 µl of this preculture were transinoculated into a main culture such that the initial OD (660 nm) of the main culture was 0.1. By transfer of IPTG-containing

medium from the preculture, the IPTG concentration in the main culture was approx. 0.5 µM/l. Medium MM was used for the main culture.

[0097] Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50g/l
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions are then added, and the $CaCO_3$ autoclaved in the dry state is added.

[0098] Culturing was carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was

added. Culturing was carried out at 33°C and 80% atmospheric humidity.

[0099] After 67 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

[0100] The result of the experiment is shown in table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM5715	7.6	13.57
DSM5715::pXK99EdeaD	12.2	16.31

[0101] This application claims priority to German Priority Document Application No. 100 47 865.4, filed on September 27, 2000. The above German Priority Document is hereby incorporated by reference in its entirety.